Solid-State Fermentation of Soybean and Corn Processing Coproducts for Potential Feed Improvement

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ABSTRACT: Two agro-industrial coproducts, soybean cotyledon fiber and distiller's dried grains with solubles (DDGS), were used as substrates to evaluate the effect of coculturing three different fungi, *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium*, on enzyme production by solid-state fermentation (SSF). When soybean fiber was used as the substrate, a maximum xylanase activity of 757.4 IU/g and a cellulase activity of 3.2 IU/g were achieved with the inoculation and incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae* for an additional 108 h. This inoculation scheme also resulted in the highest xylanase activity of 399.2 IU/g compared to other fungi combinations in the SSF of DDGS. A large-scale SSF by this fungus combination produced fermented products that had xylanase and cellulase activities of 35.9−57.0 and 0.4−1.2 IU/g, respectively. These products also had 3.5−15.1% lower fiber and 1.3−4.2% higher protein contents, suggesting a potential feed quality improvement.

KEYWORDS: cellulase, distiller's dried grains with solubles, solid-state fermentation, soybean cotyledon fiber, xylanase

■ INTRODUCTION

Solid-state fermentation (SSF) is a fermentation process in which microorganisms are grown on solid substrate without the presence of free liquid.[1] SSF has been studied intensively for the production of enzymes, antibiotics, surfactants, and other value-added products.[2] Compared to the submerged fermentation in which nutrients are present in dissolved form in a large amount of water, SSF shows a great commercial potential due to its lower wastewater production and operating expenses, simpler fermentation medium requirement, superior productivity, and easier prevention of bacterial contamination.[3,4]

Various agro-industrial residues, such as sugar cane bagasse, cereal straws, brewer's spent grain, and corn stover, have been used as substrates for SSF to maximize their utilizations and to address the waste disposal issues. The enzyme-assisted aqueous extraction processing (EAEP) of soybeans developed by Iowa State University's Center for Crops Utilization Research (CCUR) is an environmentally friendly method for soybean oil extraction. EAEP produces soybean cotyledon fiber and soy skim as coproducts.[5] The low-valued soybean cotyledon fiber has high fiber content, and this limits its use for nonruminant feed. Thus, SSF may have a great potential in producing enzymes to break down its fiber to improve its digestibility. In addition, conventional dry-grind corn ethanol fermentation produces a significant amount of distiller's dried grains with solubles (DDGS) as a coproduct. DDGS contains high amounts of protein and fiber and is commonly used in ruminant feed. The soy liquid skim from EAEP contains partially hydrolyzed protein, and it has been shown to be a good nutrient and water source for corn ethanol fermentation.[6] The research showed an increased ethanol production rate as well as a final DDGS product with higher protein contents. The DDGS produced with such skim incorporation is referred to as soy-enhanced DDGS. The use of DDGS and soy-enhanced DDGS as nonruminant feeds should be facilitated if the fiber content could be reduced by the enzymes produced by fungi through SSF.

As SSF occurs when microorganisms grow on solid materials without the presence of free water, it can only be carried out by a limited number of microorganisms. Fungi are well adapted to SSF as their hyphae can grow on particle surfaces and penetrate into the interparticle spaces and, thereby, colonize solid substrate.[7] Three different fungi, *Aspergillus oryzae*, *Trichoderma reesei*, and *Phanerochaete chrysosporium*, were chosen for the SSF of soybean cotyledon fiber, DDGS, and soy-enhanced DDGS on the basis of their capability in producing enzyme, mostly xylanase and cellulase. *A. oryzae* has been studied for its ability to produce different enzymes by SSF of agro-industrial residues. *T. reesei* has also been used widely for its cellulase production and is commonly used in SSF studies. White rot fungi such as *P. chrysosporium* are known to secrete cellulase and xylanase enzymes,[8,9] and they are also capable of producing lignin-degrading enzyme.[10]

Coculturing of *T. reesei* and different *Aspergillus* species in SSF has been shown to enhance xylanase[11,12] and cellulase production.[13−16] The action of multiple enzymes and the interaction among different fungi are believed to be necessary to decompose complex substrates.

SSF of soybean cotyledon fiber and DDGS with the three individual fungi *A. oryzae*, *T. reesei*, and *P. chrysosporium* were previously investigated by Yang et al.,[17] but the effect of coculturing using various combinations of the fungi was unknown. Thus, the objectives of this study were (1) to investigate if there is any synergistic effect among the three fungi based on the xylanase and cellulase production, (2) to determine the best combination of fungi for large-scale SSF,

and (3) to examine the compositional change in soybean cotyledon fiber, DDGS, and soy-enhanced DDGS after large-scale SSF.

MATERIALS AND METHODS

Microorganisms, Medium, and Culture Preparation. *A. oryzae* (ATCC 1003), *T. reesei* (ATCC 13631), and *P. chrysosporium* (ATCC 24725) were provided by Professor Hans van Leeuwen of the Civil, Construction and Environmental Engineering Department, Iowa State University. All three microorganisms are generally recognized as safe[18−20] and suitable for animal feed applications. The strains were cultured on potato dextrose agar plates, and the plates were incubated at 30 °C for 7 days until complete sporulation. The spores from the plates were suspended in 15% sterile glycerol in water. The suspensions were used as cultures and were kept in vials at −20 °C until use.

Substrate Preparation and Chemicals. Soybean cotyledon fiber (6.4% protein dwb) was produced in the pilot plant of CCUR, Iowa State University, via two-stage counter-current EAEP.[5] To obtain DDGS or soy-enhanced DDGS, the first batch of corn ethanol fermentation was performed in the Iowa State University BioCentury Research Farm according to the conventional method[21] with 100% water. A second batch of corn fermentation was performed under similar conditions except that 50% of the water was replaced with soy skim fraction that was obtained from EAEP. Following the corn ethanol fermentation, downstream concentration, and drying in the pilot plant, DDGS (34.3% protein dwb) and soy-enhanced DDGS (44.5% protein dwb) were produced. The schematic diagram of this integrated soybean−corn biorefinery system is shown in Figure 1. The initial enzyme activities and composition of all three substrates were measured as described below.
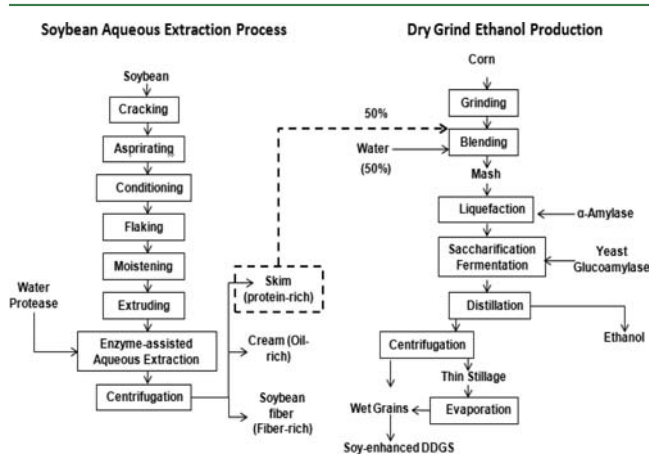

Figure 1. Schematic diagram of an integrated soybean−corn biorefinery system.

All chemicals and medium ingredients were purchased from Fisher Scientific (Pittsburgh, PA, USA), Sigma Chemicals (St. Louis, MO, USA), or BD (Franklin Lakes, NJ, USA). Soybean hulls were provided by MicroSoy Corp. (Jefferson, IA, USA).

Solid-State Fermentation. Two culture loops of *A. oryzae*, *T. reesei*, and *P. chrysosporium* were transferred to 250 mL Erlenmeyer flasks containing 50 mL of yeast extract peptone dextrose medium made of 2% yeast extract, 1% peptone, and 2% dextrose. The flasks were shaken at 150 rpm in a MAXQ Mini 4450 orbital shaker (Thermo Scientific, Asheville, NC, USA) at 30 °C for 24 h for *A. oryzae* and *T. reesei* and for 48 h for *P. chrysosporium*. An inoculum of each fungus at 5% (v/w) was used to inoculate 40 g (as-is) of substrates in mixed fungus culture, whereas a 10% (v/w) inoculum was used in the single-fungus culture. Soybean fiber with an original moisture content of 85% was adjusted to a moisture content of 75% by using soybean hulls, and the pH was adjusted to 5.0 as reported.[17]

DDGS was hydrated to 85% of moisture and was adjusted to a moisture content of 75% with soybean hulls, using the same amount as for soybean fiber. Soybean hulls improve the porosity of substrates for better fungal growth as demonstrated in our previous study.[17] The pH of DDGS was adjusted to 5.0 as well. SSF was done by incubating the substrates at 30 °C for 6 days. No nutrients or minerals were supplemented to the substrates in this study. All of the substrates were sterilized by holding them at 121 °C and 103 kPa in an autoclave for 20 min. The number of replicates used for each treatment is shown in each section below. A water reservoir was placed in the incubator to maintain the relative humidity and, thus, moisture level of the substrate.

Effect of Dual and Triple Fungi Combination SSF on Enzyme Activities of Soybean Cotyledon Fiber. Fungi were inoculated in dual and triple combinations to investigate if there is synergistic effect among the fungi. Soybean fiber was inoculated with a single fungus as a comparison. Xylanase and cellulase activities of the fermented soybean fiber were measured after 6 days of SSF at 30 °C. All treatments were repeated two times.

Effect of Different Inoculation Times of A. oryzae and T. reesei. Due to the absence of synergistic effect among different fungi when inoculated at the same time, inoculation times of *A. oryzae* and *T. reesei* were adjusted to determine the effect of inoculation sequence on enzyme activities. *A. oryzae* was inoculated at time 0, followed by inoculation of *T. reesei* to the 36 h *A. oryzae* fermented soybean fiber, which was expressed as *A 36h+T*. Selection of 36 h as incubation time was based on previous studies.[13,14] Treatment with *T. reesei* inoculation and incubation for 36 h, followed by inoculation of *A. oryzae*, expressed as *T 36h+A*, was investigated as well. Treatments of single-fungus inoculation with *A. oryzae* and *T. reesei* and coculture inoculation with *A. oryzae* and *T. reesei* (*A+T*) at the same time were used as comparison. Xylanase and cellulase activities were measured. All treatments were repeated three times.

Effect of Different Inoculation Times of P. chrysosporium and T. reesei. The inoculation sequence of *T. reesei* and *P. chrysosporium* was tested in the same manner as the SSF with *A. oryzae* and *T. reesei*. All treatments were repeated three times.

Effect of Different Inoculation Times of A. oryzae and P. chrysosporium. For *A. oryzae* and *P. chrysosporium* with different inoculation sequences, the same approach was used as for the combinations above. All treatments were repeated three times.

Effect of Different Inoculation Time of A. oryzae, T. reesei, and P. chrysosporium. To confirm and further compare the results obtained from different inoculation sequences with various dual fungus combinations, combinations with the expected high enzyme activities from previous trials were made and evaluated. Two new treatments were used, which were incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae*, expressed as *(T&P) 36h+A*, and incubation of *T. reesei* for 36 h, followed by *A. oryzae* and *P. chrysosporium*, expressed as *T 36h+(A&P)*. For comparison, single-culture inoculation of *T. reesei* and *T. reesei* inoculation for 36 h, followed by *A. oryzae* (*T 36h+A*) were also included. All treatments were repeated three times.

Effect of Dual and Triple Fungus Combination SSF on Enzyme Activities of DDGS. SSF with the DDGS substrate was conducted by using different dual and triple fungus inoculations with simpler design based on the results from the previous batches of soybean fiber SSF. Single-fungus inoculations were included in the same batch of SSF as comparisons. On the basis of soybean fiber SSF observation and other studies, treatment with inoculation of *T. reesei* and *A. oryzae* (*T+A*) was replaced by incubation of *T. reesei* for 36 h, followed by inoculation of *A. oryzae* (*T 36h+A*). The best fungus combination from soybean fiber SSF that had the highest enzyme activity, *(T&P) 36h+A*, was used in the SSF of DDGS. Xylanase and cellulase activities of the fermented DDGS were measured after 6 days of SSF at 30 °C. All treatments were repeated three times.

Effect of Large-Scale SSF on Enzyme Activities and Composition Change in the Three Fermented Materials. To produce large quantities of solid-state fermented materials for preliminary feeding trials, large-scale SSF (300 g, as-is) of soybean

Table 1. Enzyme Activity and Composition of Soybean Cotyledon Fiber, DDGS, and Soy-Enhanced DDGS

	enzyme activities (IU/g)		composition (%, dwb)						
	xylanase	cellulase	hemicellulose	cellulose	lignin	oil	protein	ash	other carbohydrate
soybean fiber	8.0	0.3	26.5	22.3	1.9	3.3	6.4	4.3	35.3
DDGS	1.9	0.2	24.9	11.6	7.8	14.0	34.3	4.3	3.2
soy-enhanced DDGS	1.7	0.3	22.9	5.5	1.6	11.8	44.5	7.2	6.5

fiber, DDGS, and soy-enhanced DDGS was conducted. Because a high amount of soybean hulls was not desirable for feeding trials, soybean cotyledon fiber was freeze-dried prior to SSF to reduce the moisture content to minimize the use of soybean hulls. The moisture content of soybean fiber and DDGS was adjusted to 50% with the addition of 5% (dwb) soybean hulls to improve their porosity. Because the moisture content of 50% in soy-enhanced DDGS was too high based on visual observation of the presence of free water, soy-enhanced DDGS was adjusted to a moisture content of 40%. All substrates had water activity above 0.90.

The pH of the substrates was adjusted to 5.0. A 5% (v/w) fungal inoculum was used to inoculate 300 g (as-is) of substrate, with the inoculation and incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by inoculation of *A. oryzae*, expressed as *(T&P) 36h+A*. This inoculation scheme was chosen from the previous batches of small-scale SSF. SSF was done by incubating the samples at 30 °C for 6 days, for the duplicate SSF of the three different substrates. Dry matter mass and xylanase and cellulase activities of the fermented substrates were measured after the SSF. All of the fermented substrates were dried at 80 °C until completely dry, and replicates of the same substrate were mixed together for fiber, protein, lipid, and ash content analyses as described below.

Enzyme Activity Assays and Fiber Content Quantification. Xylanase and cellulase activities were assayed as reported by Yang et al.[17] Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) analyses were performed according to the methods of Goering and Van Soest.[22] All samples were analyzed in duplicate.

Determination of Dry Matter Mass, Protein, Lipid, and Ash Contents. The dry matter mass was determined by weighing after oven-drying at 105 °C overnight. Protein content was determined using the Dumas nitrogen combustion method using an Elementar-Vario MAXCN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) with a conversion factor of 6.25, and lipid content was determined by an acid hydrolysis method (AOAC official method 922.06). Ash content was determined by heating the samples at 550 °C overnight (AOAC official method 923.03). The other carbohydrate content was obtained by subtracting the protein, lipid, fiber, and ash contents from 100%.

Statistical Analysis. All treatments were repeated as described under each experiment. The data were analyzed by Analysis of variance (ANOVA) using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA), and the least significant difference (LSD) mean comparison was used to compare the treatment mean differences at $P = 5\%$.

RESULTS AND DISCUSSION

Effect of Dual and Triple Fungus Combination SSF on Enzyme Activities of Soybean Cotyledon Fiber. The enzyme activities of unfermented soybean fiber were assayed as control, and it had xylanase activity of 8.0 IU/g and cellulase activity of 0.3 IU/g as shown in Table 1. Figure 2 shows both xylanase and cellulase activities among different combinations of fungi after SSF at 30 °C. Soybean fiber with mixed inoculation of *T. reesei* and *P. chrysosporium* had higher acitivity for both enzymes compared to other mixed-fungus inoculated samples. However, its xylanase activity was lower than that of the *T. reesei* inoculation alone. Fermented soybean fiber with other combinations of fungi all showed lower activity for both enzymes compared to the single-fungus inoculation. The low
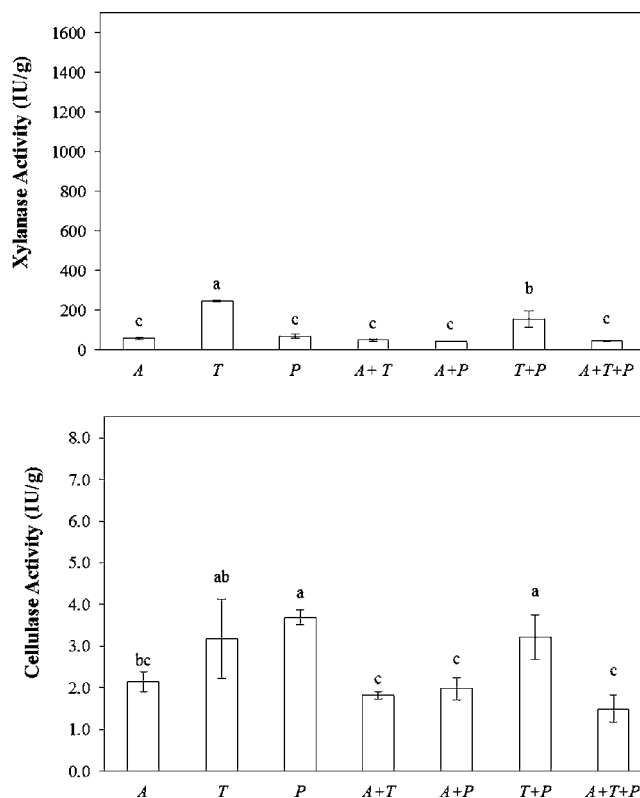

Figure 2. Effect of different fungi and combination on enzyme activities of soybean cotyledon fiber. $N = 2$. Means followed by the same letter are not significantly different at $P = 5\%$. $LSD_{0.05} = 39.6$ for xylanase activity, and $LSD_{0.05} = 1.1$ for cellulase activity. A, *A. oryzae*; T, *T. reesei*; P, *P. chrysosporium*.

enzyme activities obtained from this mixed-fungus culture SSF was unexpected, especially with the combination of *T. reesei* and *Aspergillus* species, which was shown to have synergistic effects in producing xylanase and cellulase.[11−16] Fungi may have responded differently to different substrate and growing conditions, and our soybean fiber SSF conditions might not be the optimum for observing the synergistic effect among fungi. On the other hand, the inoculation sequence of different fungal strains might play a significant role in stimulating the enzyme production. Castilo et al. reported that a mixed fungal inoculation sequence of *T. reesei* and *A. niger* must be adjusted carefully due to the differences in individual fungal growth rates, enzyme production rates, and the possible dominance of one fungus over the other.[11]

Effect of Different Inoculation Times of A. oryzae and T. reesei. Investigations on different inoculation times of *A. oryzae* and *T. reesei* were conducted to determine the effect on enzyme production. Because Dueñas et al.[23] reported that mixed culturing was not beneficial when *T. reesei* and *Aspergillus phoenicis* were inoculated together and knowing that *T. reesei* produces reducing sugars through cellulose hydrolysis that may accelerate the growth of *Aspergillus*, we conducted sequential

inoculations. As shown under experiment 1 in Figure 3, a xylanase activity of 1445.7 IU/g was obtained in the fermented
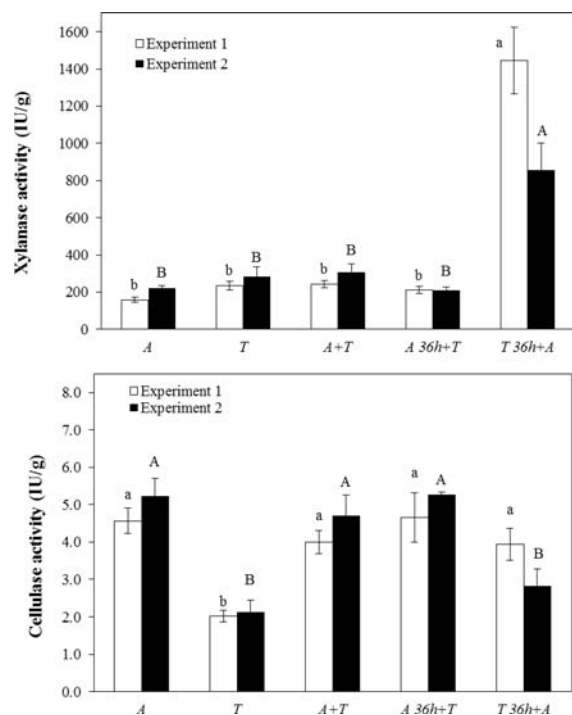

Figure 3. Effect of different inoculation times of *A. oryzae* and *T. reesei* on enzyme activities of soybean cotyledon fiber. $N = 3$. Means followed by the same letter within each experiment are not significantly different at $P = 5\%$. $LSD_{0.05} = 148.2$ for xylanase activity and $LSD_{0.05} = 0.7$ for cellulase activity in experiment 1. $LSD_{0.05} = 131.7$ for xylanase activity and $LSD_{0.05} = 0.8$ for cellulase activity in experiment 2. *A*, *A. oryzae*; *T*, *T. reesei*; *A 36h+T*, *A. oryzae* for 36 h, followed by *T. reesei*; *T 36h+A*, *T. reesei* for 36 h, followed by *A. oryzae*.

soybean fiber with the inoculation of *T 36h+A*, and this activity was significantly higher than all the other treatments. To validate the synergistic effect observed, the SSF experiment with the same treatments was repeated and the results are presented as experiment 2 in Figure 3. Again, the highest xylanase activity of 855.8 IU/g was obtained in the fermented soybean fiber with the inoculation of *T 36h+A*. Xylanases are mainly produced by *Aspergillus* and *Trichoderma* spp.[24] Our findings showed that a synergistic effect occurred when these two fungi were inoculated with an appropriate sequence that stimulated higher xylanase production. Besides xylan (hemicellulose), studies have shown that xylanase activity could be induced when *T. reesei* was grown in cellulose.[16] Our soybean fiber contained high levels of both hemicellulose (26.5%) and cellulose (22.3%), so this may explain the high level of xylanase activity in soybean fiber inoculated with *T. reesei*, as well as the mixed culture of *T. reesei* and *A. oryzae* with a proper inoculation sequence. Unlike the xylanase activiy, no significant synergism was found in the cellulase activities with the mixed fungi of *A. oryzae* and *T. reesei*. Although a basal medium of Mandal and Reese[25] containing all nutrients has been commonly added to solid substrates to give optimum fungal growth and increase the cellulase production, we did not add any external nutrients in this experiment. We intended to test if this substrate alone can simply be solid-state fermented to improve its compositional profile.

Both xylanase and cellulase activities of *A. oryzae* fermented soybean fiber from Figure 2 were lower than the enzyme activities shown in Figure 3. Also, inconsistency was noted in enzyme activities of *P. chrysosporium* fermented soybean fiber shown in Figure 2 compared to the enzyme activies obtained from other batches of SSF as discussed below. Such inconsistencies were unexpected, and they might be caused by the viability variance among samples in the individual inoculum vials, handling errors, and the complexity of the biological system.

Effect of Different Inoculation Times of P. chrysosporium and T. reesei. To determine if different inoculation times would result in a synergistic effect with the fungus combination of *P. chrysosporium* and *T. reesei*, SSF of soybean fiber with these two fungi inoculation was done. As shown under experiment 1 in Figure 4, fermented soybean fiber with the inoculation of *T+P*
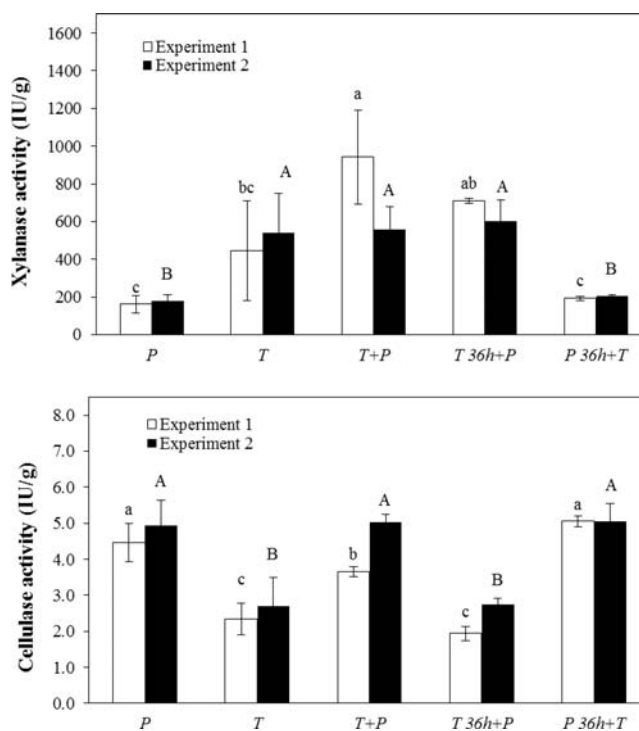

Figure 4. Effect of different inoculation times of *P. chrysosporium* and *T. reesei* on enzyme activities of soybean cotyledon fiber. $N = 3$. Means followed by the same letter within each experiment are not significantly different at $P = 5\%$. $LSD_{0.05} = 299.4$ for xylanase activity and $LSD_{0.05} = 0.6$ for cellulase activity in experiment 1. $LSD_{0.05} = 219.3$ for xylanase activity and $LSD_{0.05} = 1.0$ for cellulase activity in experiment 2. *T 36h+P*, *T. reesei* for 36 h, followed by *P. chrysosporium*; *P 36h+T*, *P. chrysosporium* for 36 h, followed by *T. reesei*.

and *T 36h+P* showed the highest xylanase activities among all fermented samples. However, these two combinations did not always contribute to the highest cellulase activities. Fermented soybean fiber with both the inoculation of *P* and *P 36h+T* had higher cellulase production. Due to the large standard deviation from this batch of SSF, another batch of SSF with the same treatments was conducted. The results shown under experiment 2 in Figure 4 indicate no significant differences among inoculation of *T*, *T+P*, and *T 36h+P* in xylanase activities. Large standard deviation was again found in enzyme activities. The lack of homogeneity in fungal growth on the substrate might have contributed to the large differences. Nonetheless, cellulase

activities measured from the repeated SSF showed a similar trend compared to experiment 1. On the basis of the results obtained from both experiments, the treatment of *T+P* was identified to have a great potential in producing high levels of xylanase and cellulase. As discussed earlier, the high hemicellulose and cellulose content in soybean fiber might help induce xylanase production in *T. reesei* inoculated substrate and also increase xylanase activity in treatment of *T+P*. The combination of *T+P* was also found to be the best in lignocellulosic decomposition of timber waste.[26] Such combination was also used in other studies in composting different solid wastes.[27,28] As a result, this combination was used in our study for further investigation.

Effect of Different Inoculation Times of A. oryzae and P. chrysosporium. SSF of soybean fiber with inoculation of *A. oryzae* and *P. chrysosporium* at different inoculation times was investigated as well. Xylanase and cellulase activities of various fungus treatments and with different inoculation times are shown in Figure 5. On the basis of the two enzyme activities, no
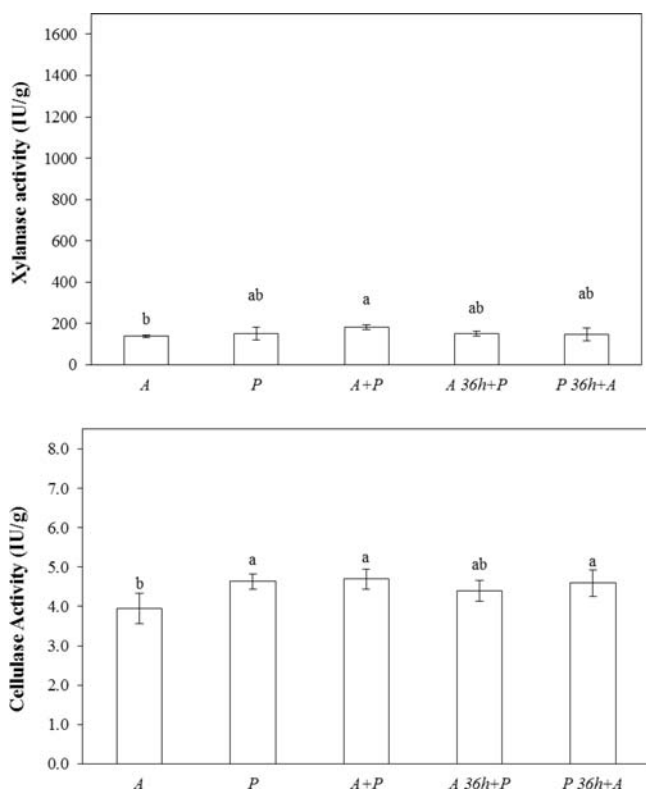
Figure 5. Effect of different inoculation times of *A. oryzae* and *P. chrysosporium* on enzyme activities of soybean cotyledon fiber. N = 3. Means followed by the same letter are not significantly different at P = 5%. LSD$_{0.05}$ = 37.9 for xylanase activity, and LSD$_{0.05}$ = 0.5 for cellulase activity. A 36h+P, A. oryzae for 36 h, followed by P. chrysosporium; P 36h+A, P. chrysosporium for 36 h, followed by A. oryzae.

significant synergistic effect was observed, and xylanase activity among these combinations was lower compared to the other two fungal combinations.

Effect of Different Inoculation Times of A. oryzae, T. reesei, and P. chrysosporium. The treatments that gave high enzyme activities from each dual combination of SSF were further combined, and such combination effects on xylanase and cellulase activities were evaluated. As shown in Figure 6, both enzyme activities from *T. reesei* inoculated soybean fiber were
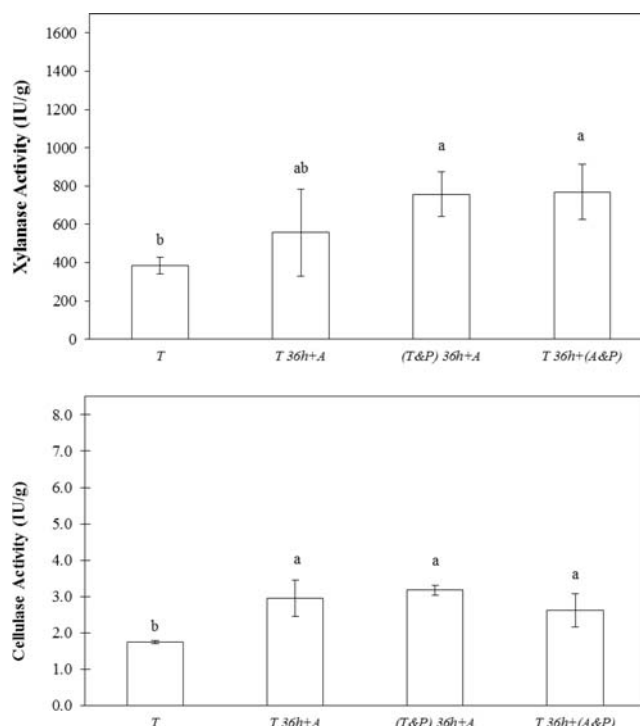
Figure 6. Effect of different inoculation times of *A. oryzae*, *T. reesei*, and *P. chrysosporium* on enzyme activities of soybean cotyledon fiber. N = 3. Means followed by the same letter are not significantly different at P = 5%. LSD$_{0.05}$ = 279.8 for xylanase activity, and LSD$_{0.05}$ = 0.7 for cellulase activity. T 36h+A, T. reesei for 36 h, followed by A. oryzae; (T&P) 36h+A, T. reesei and P. chrysosporium for 36 h, followed by A. oryzae; T 36h+(A&P), T. reesei for 36 h, followed by A. oryzae and P. chrysosporium.

the lowest among all of the treatments. No significant difference was found in xylanase activities among the three combinations, *T 36h+A*, *(T&P) 36h+A*, and *T 36h+(A&P)*. If *P. chrysosporium* is considered to be a lignin-degrading enzyme producer,[29,30] the combination including this fungus should be chosen because soybean fiber contains lignin. In addition, lignin degradation enables the exposures of hemicellulose and cellulose for degradation. Summarizing data gathered from all previous batches of soybean fiber SSF, the combination of *(T&P) 36h+A* was chosen as the final best fungus combination.

Our soybean cotelydon fiber SSF with the inoculation of *T. reesei* did not produce a significant amount of cellulase, although *T. reesei* has been reported as a promising strain in producing cellulase. The cellulolytic activity of *T. reesei* wild-type strain (QM 6a) that was used in our study has been improved by different mutagenesis methods that have resulted in mutants such as QM 9414 and RUT-C30 that can produce 4−5 times more cellulase than the wild-type strain.[31] This might partially explain the low cellulase activity of the mixed-fungal fermentation using *T. reesei* compared to other studies that employed *T. reesei* mutants.

Some batch to batch differences in enzyme aciticities were found in the fermented soybean fiber. These may be caused by the lack of homogeneity in the substrate, soybean cotyledon fiber, that was produced from pilot-plant scale EAEP.[5] Approximately 45 kg (as-is) of soybean fiber was produced in each trial of EAEP, and the hand-mixing might not have been adequate to obtain a homogeneous mixture for all treatments and replicates. Having a more effective means of mixing during

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Table 2. Effect of Different Fungus Combinations on Enzyme Activity of DDGS[a]

	fungus combination								
	A	T	P	T 36h+A+	T+P	A+P	(T&P) 36h+A	A+T+P+	LSD$_{0.05}$
xylanase activity (IU/g)	88.3 ± 5.1 f	218.3 ± 24.6 d	364.8 ± 22.3 b	293.1 ± 7.8 c	157.0 ± 25.3 e	114.8 ± 5.7 f	399.2 ± 31.4 a	120.0 ± 11.5 f	33.4
cellulase activity (IU/g)	2.1 ± 0.5 b	2.3 ± 0.7 b	7.3 ± 0.4 a	1.8 ± 0.2 b	2.0 ± 0.4 b	1.9 ± 0.4 b	2.0 ± 0.2 b	1.7 ± 0.1 b	0.7

[a] $N = 3$. Data are presented as the mean ± SD. Values followed by the same letter in the same row are not significantly different at $P = 5\%$. A, *A. oryzae*; T, *T. reesei*; P, *P. chrysosporium*. All of the different sequences are described in Figure 6.

production or fermentation in the future may help to minimize the variability in SSF.

Effect of Dual and Triple Fungus Combination SSF on Enzyme Activities of DDGS. The enzyme activities of unfermented DDGS were assayed as control and are shown in Table 1. Inoculation with different fungus combinations for DDGS SSF was done in a simpler way by using the results from soybean fiber SSF. The two enzyme activities of the fermented substrates are presented in Table 2. The fermented sample with the inoculation of *(T&P) 36h+A*, which was the best combination for soybean fiber SSF, showed the highest xylanase activity compared to the inoculation with a single fungus and with other fungus combinations. Meanwhile, the fermented DDGS with inoculation of *P. chrysosporium* was identified to produce a high xylanase activity of 364.8 IU/g. This is appreciably higher than all of the batches of soybean fiber SSF inoculated with *P. chrysosporium*. In addition, this sample also showed the highest cellulase activities of 7.3 IU/g. This suggests that in contrast to soybean fiber, DDGS might have a better composition to support the growth of and enzyme production from *P. chrysosporium* in SSF. According to Leštan et al.,[32] the addition of linseed oil to the growth medium strongly stimulated mycelium biomass, production of *P. chrysosporium*. DDGS has higher content of lipid compared to soybean fiber as shown in Table 1, and this may explain the better growth of *P. chrysosporium* in DDGS, thus contributing to the higher enzyme production. Due to the high hemicellulose content of 24.9% compared to the low cellulose content of 11.6% in DDGS, xylanase activity is considered to be more important in breaking down the substrate. Therefore, inoculation of *(T&P) 36h+A* was chosen as the best combination for further study. This same best fungus combination as for soybean fiber allows the future comparison of various substrates under the same fermentation condition.

Effect of Large-Scale SSF on Enzyme Activities and Composition Change in the Three Fermented Materials. The 300 g scale SSFs of soybean fiber, DDGS, and soy-enhanced DDGS with the best fungus combination, *(T&P) 36h+A*, were conducted, and xylanase and cellulase activities of the fermented materials are shown in Table 3. Compared to the SSF of soybean fiber and DDGS with the same fungus inoculation that were done on small scale (40 g), the enzyme productions from the large-scale SSF were much lower. The lower amount of soybean hulls (5%, dwb) added in the substrates for the 300 g scale SSF may have contributed to the lower enzyme activities because all enzyme activities are presented as IU for 1 g of dried substrate without the inclusion of the soybean hulls. The higher amount of soybean hulls (47%, dwb) used previously in the substrates for small-scale SSF and the lower amount of dried soybean fiber or DDGS were included in the calculation, thus contributing to the higher enzyme activities. The other factor may be that the lower

Table 3. Effect of SSF Using the Best Combination of Fungi on Enzyme Activity of Soybean Cotyledon Fiber, DDGS, and Soy-Enhanced DDGS[a]

	soybean fiber	DDGS	soy-enhanced DDGS	LSD$_{0.05}$
xylanase activity (IU/g)	57.0 ± 9.7 a	49.3 ± 3.0 ab	35.9 ± 2.2 b	19.1
cellulase activity (IU/g)	1.2 ± 0.1 a	0.6 ± 0.0 b	0.4 ± 0.1 c	0.2

[a] $N = 2$. Data are presented as the mean ± SD. Values followed by the same letter in the same row are not significantly different at $P = 5\%$.

amount of soybean hulls used in the large-scale SSF caused a compact texture of the substrates, thus reducing the fungal growth and hyphae penetration. As shown in Table 3, both enzyme activities indicated a similar trend, with fermented soybean fiber having the highest activities, followed by DDGS and soy-enhanced DDGS. However, the xylanase activities of soybean fiber and DDGS were not significantly different due to the large standard deviation found in fermented soybean fiber. Consistent with the results from Yang et al.,[17] soybean fiber is shown to be a better substrate for SSF compared to DDGS. This may be due to the limited nutrient availability in DDGS for fungal growth. The high amount of the other carbohydrate content in soybean fiber (Table 1) compared to DDGS and soy-enhanced DDGS could be a good carbon source to support the growth of fungi and to produce enzyme. As shown in Table 3, soy-enhanced DDGS demonstrated low enzyme activities after SSF. The high ash content in soy-enhanced DDGS as shown in Table 1 indicates the high level of salt, and this may be a reason for its poor fungal growth. Salt was shown to have adverse effects on microorganisms and resulted in reduced biological activity.[33]

Substrate weight reduction for 300 g scale fermentation was recorded. An approximately 20.4% reduction in dry matter mass was found in fermented soybean fiber, followed by reductions of 9.9% in DDGS and 6.4% in soy-enhanced DDGS. The dry matter reduction reflected the vigor of fungus growth, with higher reduction indicating better fungal growth. The dry matter reduction of the three fermented samples correlated well with the enzyme production.

Table 4 shows the composition of unfermented and fermented soybean fiber, DDGS, and soy-enhanced DDGS. The theoretical values are those with the disappearance of dry matter taken into consideration. Fiber content decreased substantially in each fermented sample with hemicellulose content showed a greater reduction compared to cellulose and lignin content. This trend corresponded well to the xylanase and cellulase activities in the fermented samples. The reductions of hemicellulose content in the fermented soybean fiber, DDGS, and soy-enhanced DDGS were 13.4, 11.5, and 4.5%, respectively. The degree of reduction is proportional to

Table 4. Composition of Unfermented and SSF Soybean Cotyledon Fiber, DDGS, and Soy-Enhanced DDGS

substrate		composition (%), dwb				
		hemicellulose	cellulose	lignin	protein	lipid
soybean fiber	unfermented[a]	22.0	25.5	1.2	8.3	3.5
	fermented	10.8	30.1	0.9	12.5	5.0
	theoretical value[b]	8.6	24.0	0.8	9.9	4.0
	increase (+) or decrease (−)	−11.2	4.5	−0.2	4.2	1.5
	theoretical increase (+) or decrease (−)	−13.4	−1.6	−0.4	1.6	0.5
DDGS	unfermented[a]	30.5	13.8	5.7	33.0	11.3
	fermented	21.0	12.8	1.1	36.3	14.6
	theoretical value[b]	18.9	11.5	1.0	32.7	13.2
	increase (+) or decrease (−)	−9.5	−1.0	−4.6	3.3	3.3
	theoretical increase (+) or decrease (−)	−11.5	−2.2	−4.7	−0.3	1.9
soy-enhanced DDGS	unfermented[a]	21.2	8.9	3.7	43.0	10.5
	fermented	17.9	10.0	2.3	44.4	11.1
	theoretical value[b]	16.8	9.4	2.2	41.5	10.4
	increase (+) or decrease (−)	−3.3	1.1	−1.3	1.3	0.6
	theoretical increase (+) or decrease (−)	−4.5	0.4	−1.5	−1.5	−0.1

[a]All of the unfermented materials contained 5% (dwb) soybean hulls. [b]Theoretical values are those based on the initial substrate weights, not the final weight-reduced substrate quantity.

the increase in xylanase activity. Cellulose contents in fermented soybean fiber and soy-enhanced DDGS increased after the SSF. The reduction in dry matter by SSF as a result of the metabolism of substrate by microorganisms may have contributed to the increase in cellulose content. By taking the dry matter reduction into consideration, the theoretical value of cellulose showed 1.6 and 2.2% decreases in fermented soybean fiber and DDGS and a slight increase of 0.4% in fermented soy-enhanced DDGS. Lignin content in all of the fermented materials decreased after SSF, with the highest reduction in DDGS, followed by soy-enhanced DDGS and soybean fiber. This finding could be explained by the high lipid content in DDGS and soy-enhanced DDGS that may have stimulated the growth of *P. chrysosporium* as discussed earlier.

Protein content showed a slight increase after SSF, and this could be explained by the concentrating effect caused by microorganisms metabolizing the substrates. The higher protein content in the fermented product is expected to improve its nutritional value as animals feed. The lipid content of the three substrates did not change significantly after SSF, unlike other papers which suggested that *Aspergillus* and *Rhizopus* metabolized a portion of fat in the DDGS.[34] Liu reported that DDGS composition varied with differences in feedstock and composition, process methods and parameters, and fermentation yeast.[35] The composition of DDGS used in this study may be different from the one used in the other study, and this may have caused the difference in fungal growth and substrate composition change. The slight increases of the total lipid content in soybean fiber, DDGS, and soy-enhanced DDGS after SSF could be from the concentration effect.

In summary, a synergistic effect was found among the three fungi used in the SSF of soybean cotyledon fiber and DDGS. The inoculation sequence of different fungi was identified as an important factor to allow the best interaction among the fungi to achieve better growth and higher enzyme production. Combination of fungi with the incubation of *T. reesei* and *P. chrysosporium* for 36 h, followed by *A. oryzae*, showed the best results in soybean cotyledon fiber SSF. The fermented soybean fiber has a maximum xylanase activity of 757.4 IU/g and a

cellulase activity of 3.2 IU/g. This inoculation scheme also led to the highest xylanase activity of 399.2 IU/g in DDGS SSF. The fermented materials produced from the 300 g scale SSF showed a 3.5−15.1% reduction in fiber content and a 1.3−4.2% increase in protein content, demonstrating the potential for nonruminant feed improvement.

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Notes
The authors declare no competing financial interest.

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